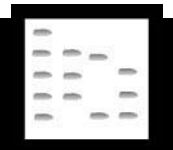
PROSTAGLANDINS: GAS CHROMATOGRAPHY



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Introduction

Metabolism of free (nonesterified) arachidonic acid (5,8,11,14-cis-eicosatetraenoic acid) via the cyclooxygenase (COX) pathway produces a cascade of biologically active compounds collectively known as prostaglandins (PGs). These include prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin F_2 (PGF_2), prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) (**Figure 1**). The analysis of PGs and related compounds in isolated cells and organs has proved relatively easy. By contrast, measurement of PGs in plasma presents a more challenging analytical task. This is because: (1) a more complex mixture of chemically related compounds exists in plasma; (2) PGs exert their effects locally, i.e. within a short distance of their site of production, and are consequently rapidly metabolized; and (3) *ex vivo* generation of PGs may occur. PG metabolites are generally present in biological fluids, including plasma and urine, in higher concentrations than their precursors. These metabolites are not formed during processing and therefore the problem of generating artefactual results does not arise. It has been suggested that the measurement of PG metabolites in urine may represent a more reliable approach for assessing *in vivo* generation of PGs than does quantification of primary products in plasma.

Recently, it has been shown that free-radical peroxidation of polyunsaturated fatty acids (PUFAs) by a mechanism independent of the COX-pathway also produces novel families of PGF-like compounds. In contrast to the COX-derived products, COX-independent products are present mainly esterified to phospholipids and with the side chains across the cyclopentane ring in the *cis* orientation. **Figure 2** illustrates the structural differences between COX-dependent and COX-independent 9α,11α-$PGF_{2\alpha}$. COX-independently derived PGF-like compounds are collectively known as isoprostanes. The measurement of isoprostanes has received considerable attention because they have proved to be a reliable marker of oxidative stress. Determination of the different families of isoprostanes also allows the assessment of the peroxidation of individual PUFAs. Accumulating data indicate that the metabolic fate of non-COX-derived PGF-like compounds is similar to that of the COX-derived products.

PGs and related compounds can be assessed by several methods including bioassay, radioimmunoassay (RIA), high performance liquid chromatography (HPLC) linked to RIA or UV or mass spectrometric (MS) detection and gas chromatography–mass spectrometry (GC-MS). GC-MS is the preferred method for the quantification and structural validation of PGs, combining the high resolution of GC separation employing fused silica capillary columns with the specificity and sensitivity of mass spectrometry.

Analysis of PGs by GC-MS

The analytical procedures for the isolation and determination of PGs in plasma and urine involve a number of steps.

Selection of an Internal Standard

The use of an internal standard is vital for accurate and precise assay of PGs and related compounds by GC-MS. An ideal internal standard should have similar physicochemical properties to the compound being analysed, and should be taken through the entire analytical procedure, i.e. extraction, enrichment and derivatization. Stable isotope-labelled internal standards are generally used for the assay of PGs and related compounds. These are prepared by replacing sufficient atoms of ^1H or ^{16}O by the corresponding stable isotopes ^2H and ^{18}O. Many tetradeuterated [$^2H_4$] PGs are commercially available. By contrast, carboxylic ^{18}O-labelled PGs have to be synthesized. The following precautions should be considered during sample processing: (1) the amount of the internal standard should be at least of the same order as that expected for the compound being analysed; (2) the possibility of back-exchange of ^2H by ^1H from water during alkaline hydrolysis or following catalytic hydrogenation should be considered; and similarly (3) back-exchange of carboxylic ^{18}O by ^{16}O in an esterase-rich environment such as plasma.

Isolation from Biological Samples

The strategies for the routine isolation of PGs and related compounds from biological samples involve

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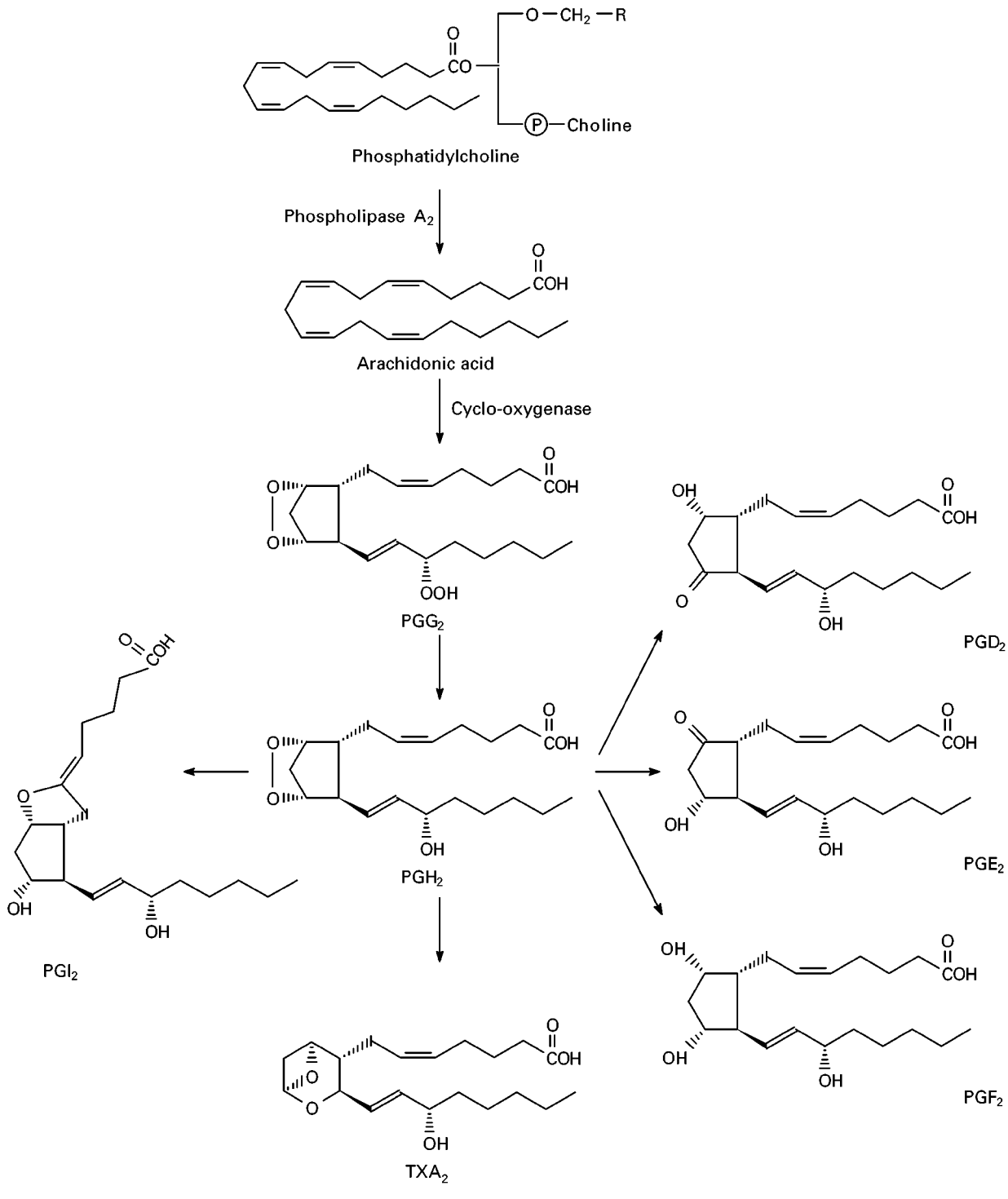

Figure 1 The release of arachidonic acid by phospholipase A_2 and its metabolism by the cyclooxgenase enzymes.

conventional solvent extraction and/or chromatographic separation involving solid-phase extraction (SPE) and thin-layer chromatography (TLC). In both situations, the samples are acidified to pH 2 to minimize ionization of the carboxylic groups of PGs. The solvent of choice for partitioning of PGs and related compounds from biological samples is ethyl acetate. SPE entails passing the sample through a disposable octadecylsilica (C_{18}) cartridge. Lipophilic compounds are retained on the column while salts and polar compounds are removed by washing the column with water or water/acetonitrile. PGs are eluted from the

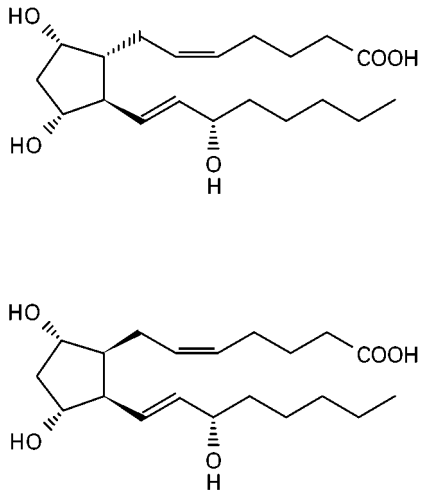

Figure 2 Structural differences between (A) COX-dependent 9α,11α-$PGF_{2\alpha}$ and (B) COX-independent 9α,11α-(8-epi)-$PGF_{2\alpha}$.

column using a combination of organic solvents such as hexane, ethyl acetate and isopropanol. The final step in the purification of PGs and related compounds involves thin-layer chromatography (TLC); one-step TLC for plasma and two-step for urine. Replacement of the C_{18} and TLC procedures with an aminopropyl-silica cartridge substantially increases the recovery of PGF-like compounds and reduces analysis time. Immunoaffinity chromatography has also been used for the purification of PGs from biological fluids. The advantages of this method over multistep procedures are: (1) its simplicity and shorter analysis times; (2) the addition of a further degree of specificity to the analysis. Unfortunately, its implementation has been limited to leading laboratories in the field because the column materials are not commercially available.

Exploiting a unique chemical property of a prostaglandin such as 2,3-dinor-6-oxo-$PGF_{1\alpha}$ is another approach for the isolation of structurally related products (**Figure 3**). 2,3-dinor-6-oxo-$PGF_{1\alpha}$ can exist in different isomeric forms depending on pH. At acid pH, the 2,3-dinor-6-oxo-$PGF_{1\alpha}$ is converted to a lactone. This property allows isolation of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ from other polar compounds such as free fatty acids. Quantitative recovery of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ is achieved by hydrolysis in a mild aqueous base.

Plasma isoprostanes are mainly present esterified to phospholipids and therefore have to be hydrolysed prior to SPE and/or TLC processing. Briefly, plasma (1 mL) is incubated with 1 mol L^{-1} aqueous potassium hydroxide at 45°C for 1 h to release esterified isoprostanes. The pH is then adjusted to 2 using HCl. The internal standard is added and the isoprostanes isolated as described above. For the analysis of isoprostanes in tissue samples, homogenates (50–100 mg tissue mL^{-1}) are hydrolysed with 1 mol L^{-1} potassium hydroxide at 45°C for 1 h. The pH is then adjusted to 2 and total lipids extracted with 10 volumes of ethyl acetate. Isoprostanes are then isolated by a single chromatography step on an aminopropyl cartridge.

Derivatization

Chemically untreated PGs are not suitable for GC analysis. Therefore, protection of functional moieties in PGs is carried out first to increase their volatility, improve chromatographic separation and to enhance assay sensitivity and specificity. The most common derivatization reactions used for PGs are: (1) esterifi-

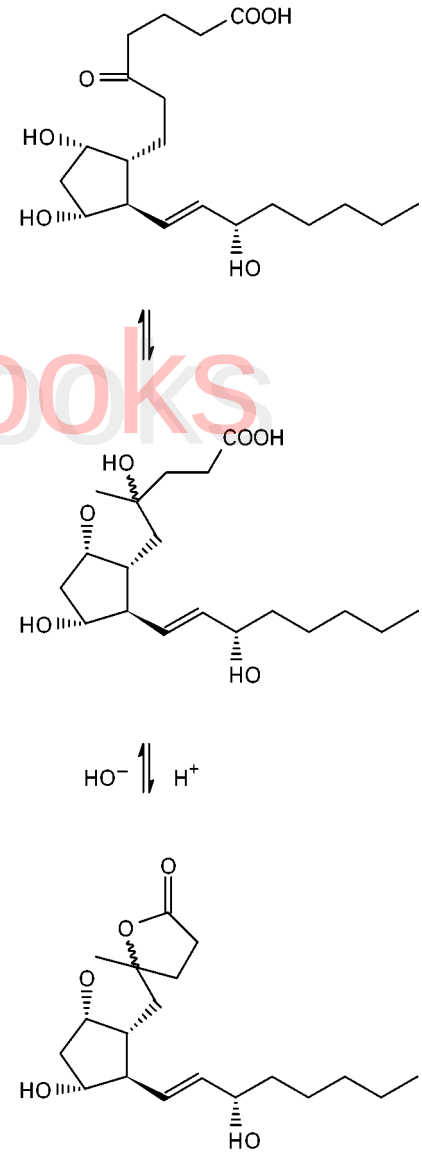

Figure 3 Equilibrium between the ketone and lactone forms of 2,3-dinor-6-oxo-$PGF_{1\alpha}$.

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cation of the carboxylic moieties; (2) etherification of hydroxy moieties; (3) methoximation of keto moieties; and (4) boronation of hydroxy moieties.

For the purposes of PG quantification by gas chromatography/negative ion chemical ionization (GC-MS/NICI), pentafluorobenzyl (PFB) ester derivatives of the monocarboxylic forms of PG are prepared. The reaction is carried out by incubating the final lipid extract in the presence of the catalyst *N*,*N*-diisopropylethylamine (DIPEA) at 45°C for 1 h. Methyl ester derivatives of PGs are prepared using diazomethane in ether solution. Methyl esters can also be prepared using methanolic solutions of hydrogen chloride or hydrochloric acid.

Hydroxy moieties are converted to trimethylsilyl (TMS) ether derivatives. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) is the most frequently used derivatization agent. A quantitative conversion to trimethylsilyl ether is achieved by incubating standards or the final lipid extract at 60°C for 1 h or overnight at 4°C in the presence of DIPEA. *t*-butyl(dimethylsilyl) (BDMS) ether derivatives are occasionally prepared as an alternative to TMS because they produce fragments of higher relative molecular mass during mass spectrometric analysis. The main disadvantages of the BDMS derivatives are: (1) bulkiness that results in incomplete derivatization of stearic-hindered hydroxy moieties; and (2) relatively long retention times.

Hydroxy groups may also be converted to alkylboronate derivatives. Alkylboronate derivatives are prepared by incubating PGs and related compounds with *n*-butylboronic acid under anhydrous conditions. These derivatives are mainly employed to determine the stereochemistry of the hydroxy moieties in the prostaglandin F (PGF)-series. Alkylboronate derivatives can only be formed if both hydroxy groups on the cyclopentane ring of a PGF molecule are *cis* orientated.

Keto moieties of PGs and related compounds are converted to *O*-alkyloximes by incubating authentic standard or buffered urine with a saturated methoxyamine hydrochloride solution in pyridine. It is important to remove the derivatization reagent completely because it causes deterioration of the chromatographic column. Chromatography on a short column of Sephadex LH-20 is therefore advisable.

Hence, derivatization is carried out in the order: methoximation, esterification, boronation and finally silylation to minimize side reactions.

Injector

The most common techniques for introducing high boiling point compounds such as PGs into the GC are programmed-temperature vaporization (PTV) and splitless injection. The PTV is designed to allow rapid heating and cooling of the injector. Briefly, sample (2 μL in iso-octane) is introduced into a temperature-controlled injector that is initially maintained at 75°C and is then raised to 85°C immediately after injection. Since iso-octane has a boiling point of 98–99°C the temperature will be sufficient to evaporate the majority of the solvent and simultaneously concentrate the sample onto the surface of the injector, which is lined with inert glass. After allowing sufficient time for removal of excess solvent, the injector temperature is rapidly raised to 275°C. This ensures that volatilization of high boiling point components occurs and permits a quantitative refocusing of the analyte at the head of the column.

The alternative method for introducing PGs into the GC, i.e. using a splitless injector, relies on cold trapping and/or a solvent effect to refocus the analyte on the head of the capillary column. Sample (2 μL in hexane) is usually introduced over a period of 30–60 s onto the column.

Column Heating

GC is performed using an oven temperature program that starts at an initial temperature of 80–100°C. The rates at which oven temperatures are increased range from 20°C min^{-1} to 30°C min^{-1}. The final temperature will be 280°C or higher, depending upon the upper limit specified for the column being employed. The time over which the column is maintained at maximum temperature (e.g. 280°C) depends upon the elution time of the compound being analysed.

Detectors

Mass spectrometry is used to detect PGs as they emerge from a GC. This approach has proved to be a flexible, sensitive and selective technique. The most popular modes of ionization employed by prostanoid researchers are described below.

Electron impact (EI) The electrons used for the ionization have an average energy of 70 eV. Initial ionization energies for most organic molecules are between 8 and 15 eV. When a molecule enters the ionization chamber the high energy causes fragmentation of the parent compound. This mode of ionization provides information on the relative molecular mass and molecular structure of the compound. Methyl ester derivatives are mainly used for GC-MS/EI. Quantitative analysis is performed by selected ion monitoring (SIM) of fragments characteristic of the compound being analysed. The concentration of a particular component in the sample is calculated by determining the ratio of intensity of a certain ion in

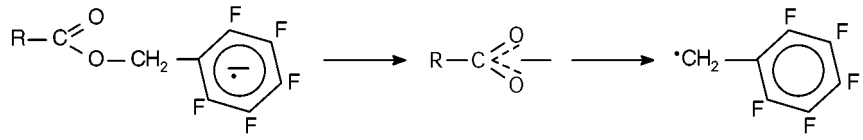

Figure 4 Formation of a carboxylated anion corresponding to the intact molecule [M-PBF]- or [M-181]- during final analysis of PFB-esters by NICI.

the sample to that of the internal standard. The detection limit for GC-MS/EI is 200 pg injected onto the column.

Positive ion chemical ionization (PICI) PICI is a softer ionization technique than EI and therefore molecules are less likely to undergo fragmentation. This leads to an abundance of protonated molecular ions. The detection limit for GC-MS/PICI is a few nanograms.

Negative ion chemical ionization (NICI) PGs and related compounds are not good electron-capturing substances. However, they contain at least one terminal carboxylic moiety that can be esterified with electron-capturing groups such as pentafluorobenzyl (PFB) esters. As illustrated in **Figure 4**, the PFB ester forms a molecular ion after initial electron capture. This leads to the loss of the PFB radical and the formation of a stabilized carboxylated anion corresponding to the intact molecule $[M\text{-}PFB]^-$ or $[M\text{-}181]^-$. For most PFB esters, the carboxylate anion constitutes $> 60\%$ of the total ion. This makes NICI a highly efficient ionization process. The combined sensitivity and selectivity of NICI has made it the method of choice for quantitative analysis of PGs. Quantitative analysis is performed using selected ion monitoring (SIM) of the carboxylate anion $[M\text{-}181]^-$. The detection limit for SIM is from 200 fg to 5 pg.

Gas chromatographic separation The most widely used stationary phase for fused silica capillary columns for the separation of PGs and related compounds is 100% dimethylsilicone commercially known as DB1, Rtx-1, SE-30, Sil-5 CB or Ultra 1. This phase has a CP-value of 5, CP-being an index that predicts selectivity and polarity for a given stationary phase. CP-values range between zero for a nonpolar stationary phase and 100 for the most polar. Other investigators have used an intermediate polarity stationary phase (CP = 19), typical commercial names being DB-1701, Rtx-1701 and Sil-19 CB. These stationary phases consist of a mixture of 86% dimethylsilicone, 7% phenylsilicone and 7% cyanopropylsilicone.

There have been problems with regard to the separation of 9α,11α-$PGF_{2\alpha}$, 9α,11β-$PGF_{2\alpha}$ and 9β,11α-$PGF_{2\alpha}$ using both low- and medium polar stationary phases. Clear separation of all three PGF-ring isomers is of interest with regard to clinical conditions linked to increased production of 9β,11α-$PGF_{2\alpha}$ such as asthma and anaphylaxis. We have found that SPB-1701 (80% dimethylsilicone, 5% phenylsilicone, 15% cyanopropylsilicone), which is considered a medium polar stationary phase with a CP-value of 20, is superior to DB-1701 for the separation of PGF_2 isomers. The improved separation of the PGF_2 isomers using the SP-1701 may be explained by the dipole moment introduced by the cyanopropyl functionality. **Figure 5** shows a baseline separation of PGF_2 isomers including 9β,11α-$PGF_{2\alpha}$, 9α,11α-(8-epi)-$PGF_{2\alpha}$, 9α,11β-$PGF_{2\alpha}$ and 9α,11α-$PGF_{2\alpha}$. The signal monitored at m/z 569 represents the carboxylated anion of the PGF_2 isomers as PFB ester/TMS ether whereas that at m/z 573 represents the tetradeuterated 9α,11α-$PGF_{2\alpha}$ internal standard.

Figure 6 shows a typical example of a SIM chromatogram obtained with simultaneous monitoring of 6-oxo-$PGF_{1\alpha}$, 2,3-dino-6-oxo-$PGF_{1\alpha}$, TXB_2, 2,3-dinor-TXB_2 and 11-dehydro-TXB_2 in human urine using a DB1 column. PGs and related compounds were purified by immunoaffinity chromatography and the final lipid extract was sequentially converted to PBF ester, methyloxime and TMS ether derivatives prior to analysis by GC-MS/NICI.

Figure 7 shows a baseline separation of *syn* and *anti* isomers of urinary PGD metabolites as PFB ester/methyloxime/TMS ether derivatives using a DB-1701 column. The peaks labelled I and II (m/z 514) represent the *syn* and *anti* isomers of urinary PGD metabolite. The signal monitored at m/z 522 is the tretraoxygen-^{18}O-labelled PGD metabolite internal standard. The peaks labelled III and IV represent a COX-independent urinary PGD-metabolite. As can be seen, *syn* and *anti* isomers derived from PGD are fully resolved by capillary gas chromatography.

There is less agreement on the best GC conditions for the separation of C_{20}-isoprostanes. C_{20}-isoprostanes are a series of PGF_2-like compounds derived from peroxidation of arachidonic acid by a mechanism independent of the COX pathway. Peroxidation

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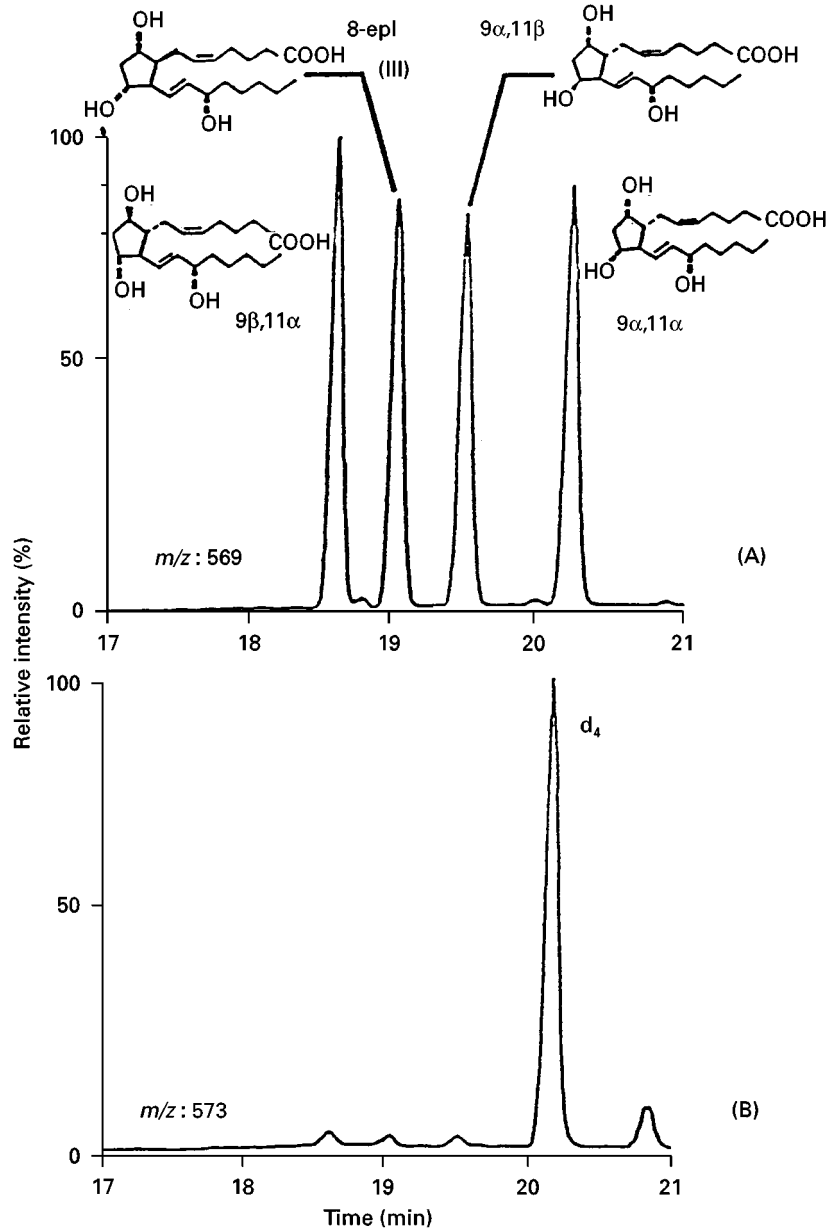

Figure 5 Gas chromatographic separation of the PGF_2-isomer as PFB ester/TMS ether derivatives using an SP-1701 column (30 m; 0.25 i.d.; film thickness 0.25 μm). (A) PGF_2-isomers; (B) tetradeuterated $PGF_{2\alpha}$ as the internal standard. (Reproduced from Nourooz-Zadeh *et al.*, 1995, with permission from Elsevier Science.)

of arachidonic acid at positions C_7, C_{10} and C_{13} would produce four families of PGF_2-like compounds. Each sub-family would have 16 diastereoisomers, since the hydroxy groups on the prostanoid skeleton can be arranged in the 2^3 configuration. Theoretically, 64 C_{20}-isoprostane isomers can be formed during the peroxidation of arachidonic acid. Of these, 9α,11α-(8-epi)-$PGF_{2\alpha}$, has received considerable attention because it is commercially available and has been shown to be biologically active. Both DB1 and DB1701 stationary phases have been used to assay C_{20}-isoprostanes in biological samples. A comparison of typical GC-MS/NICI chromatograms for 9α,11α-(8-epi)-$PGF_{2\alpha}$ in human plasma using DB-1701 and SP-1701 is shown in **Figure 8**. Once again, the SP-1701 phase is superior to the DB-1701 phase in separating the *R* and *S* isomers of 9α,11α-(8-epi)-$PGF_{2\alpha}$. To the best of our knowledge, no chromatograms have been published for the analysis of C_{20}-isoprostanes as PFB esters/BDMS in human plasma.

The SP-1701 stationary phase is also useful for the separation of C_{20}- and C_{22}-isoprostanes (PGF_3 and

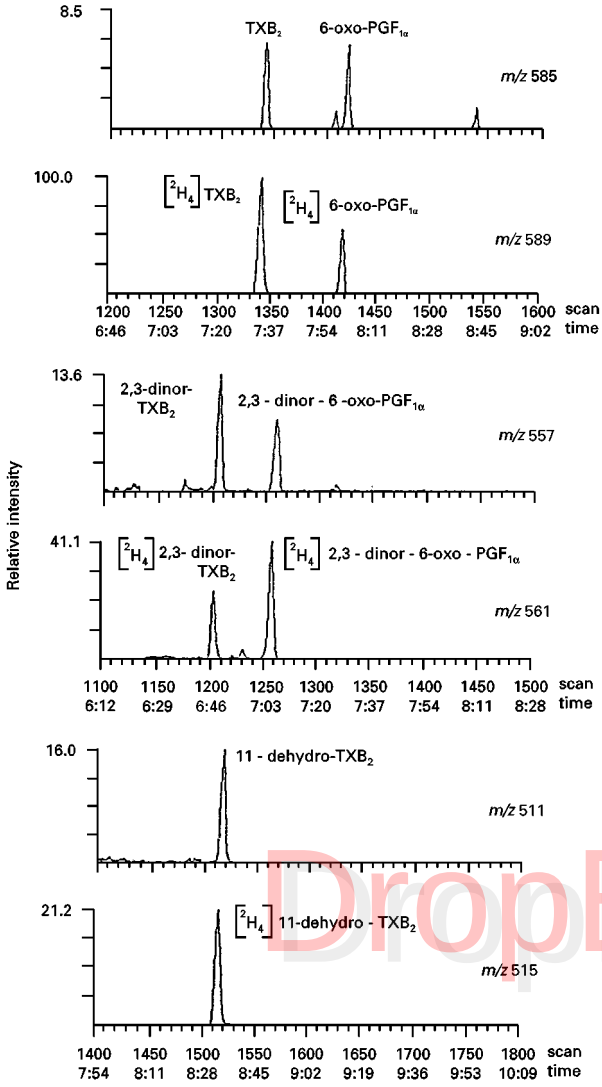

Figure 6 Selected ion monitoring chromatograms of simultaneous analysis of urinary 6-oxo-$PGF_{1\alpha}$, 2,3-dinor-6-oxo-$PGF_{1\alpha}$, TXB_2, 2,3-dinor TXB_2 and 11-dehydro-TXB_2 using a DB1 column (30 m; 0.25 i.d.; film thickness 0.25 μm). (Reproduced with permission from Barrow *et al.* (1989) *Biochimica Biophysica Acta* 993: 121–127.)

PGF_4-like compounds, respectively) in biological samples. The order of elution for the isoprostanes is C_{20}-PGF_2 followed by C_{20}-PGF_3 followed by C_{22}-PGF_4, with clear separation between the different species. Therefore, this unique property of the SP-1701 phase facilitates the parallel determination of all three classes of PGF-like compounds in a single sample. A typical GC-MS/NICI chromatogram for the separation of PGF_2- and PGF_4-like compounds in human brain tissue is shown in **Figure 9**. The upper spectrum monitored at m/z 569 represents C_{20}-PGF_2-like compounds. The middle at m/z 593 represents C_{22}-PGF_4-like compounds, while the lower spectrum at m/z 573 is the internal standard.

Identification of Compounds Based on their Retention Times

Reproducibility of chromatographic separation is important for the identification of a given compound in biological samples. For routine purposes, identification of a particular component is based on its retention time relative to that of an isotope-labelled internal standard. During GC separation on capillary columns, ^{2}H-labelled internal standard elutes 1–2 s earlier than the corresponding unlabelled compound. Carboxylic ^{18}O-labelled internal standard appears at the same retention time as the corresponding unlabelled PGs or their metabolites. Co-injection of a mixture of the samples of interest with an appropriate amount of external standard also adds a degree of confidence to the identification of the compounds based on their relative retention times.

Preparation of a Standard Curve for Quantification

A calibration curve for a given compound is prepared by plotting the peak height ratio of the unlabelled prostanoid/labelled analogue against the concentration of the compound being analysed. Such plots are generated using least-squares linear regression analysis and provide a gradient and intercept for the determination of the concentration of the corresponding unlabelled component in the sample.

Inter- and Intra-Assay Coefficients of Variation

These are determined by adding a known amount of an external standard to a sample that contains the compound of interest at a low concentration. At least four samples are required to provide an intra-assay coefficient of variation (COV). To establish the interassay COV, a minimum of four samples should be prepared on at least four occasions with different calibration curves.

Conclusions

The use of multistep chromatographic procedures in combination with GC separation on a medium polar capillary column and electron capture detection is the best approach for the analysis of PG and related compounds in biological samples. The method can be used for a wide range of lipid peroxidation products formed by mechanisms dependent or independent of the COX pathway. This is particularly important in

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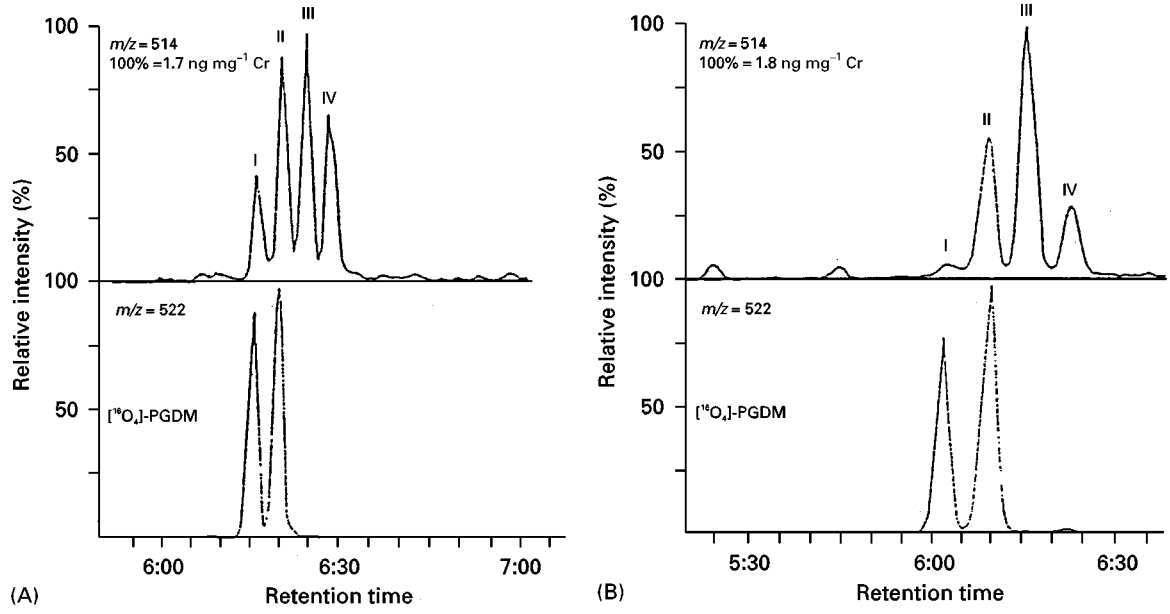

Figure 7 Selected ion monitoring chromatograms of analysis of urinary PGD metabolites using a DB1701 column (15 m; 0.25 inner diameter; film thickness 0.25 μm). (Reproduced with permission from Morrow JD *et al.* (1991) *Analytical Biochemistry* 193: 142–148).

the measurement of urinary isoprostanes and their precursors because nonenzymatic peroxidation of individual PUFAs produces an array of PGF-like compounds. The sensitivity and specificity of the assay can be improved by using GC-MS/MS or GC-tandem MS.

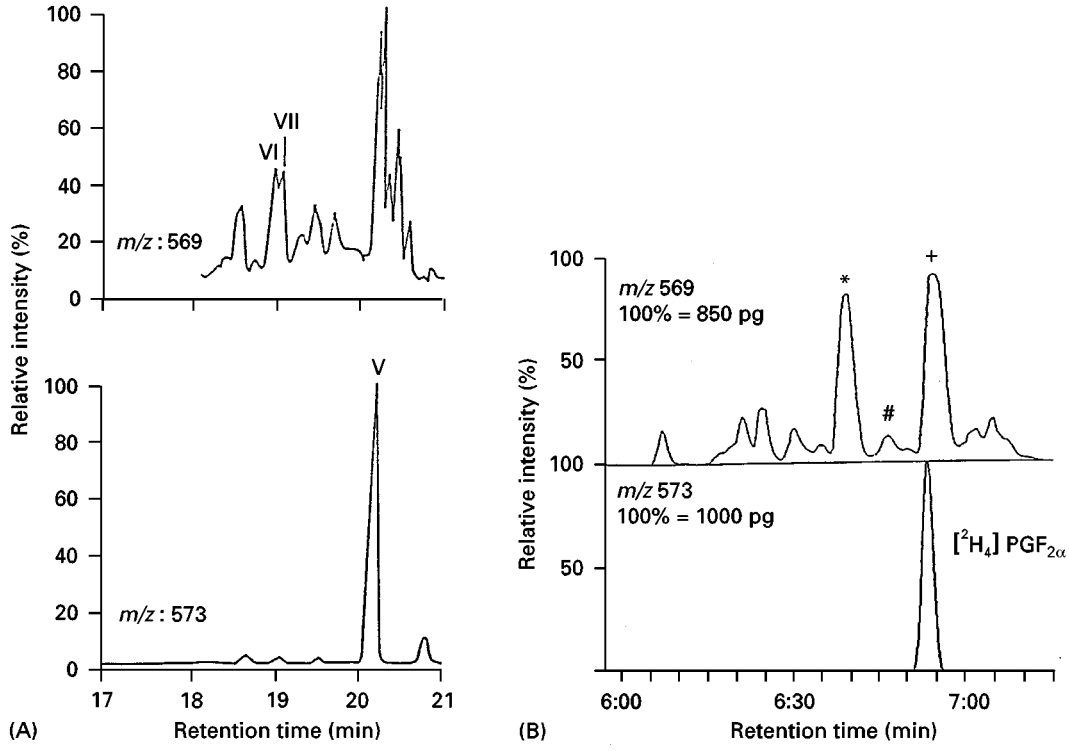

Figure 8 Comparison of chromatographic profiles of C_{20}-PGF_2-isoprostanes in human plasma using DB-1701 and SP-1701 stationary phases. (Part (A) reproduced with permission from Nourooz-Zadeh J (1999) *Methods in Enzymology* 300: 13–17; part (B) reproduced with permission from Morrow JD *et al.* (1994) *Methods in Enzymology* 233: 163–174).

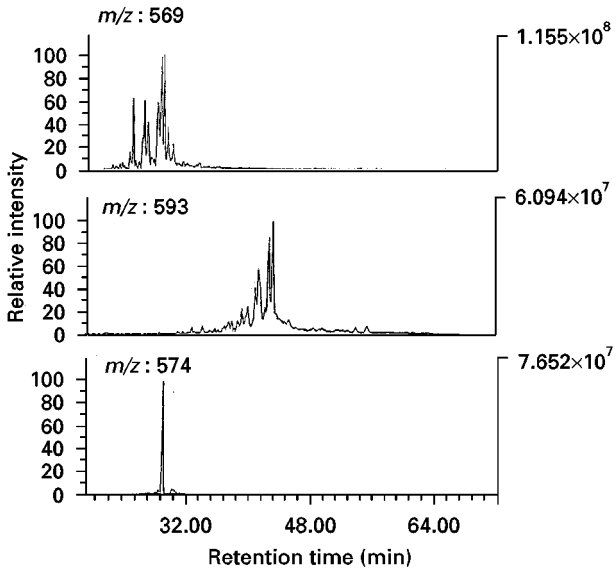

Figure 9 Selected ion monitoring chromatograms of simultaneous analysis of C_{20}-PGF_2- and C_{22}-PGF_4-isoprostanes in human brain.

See Colour Plate 115.

See also: **II/Chromatography: Gas:** Column Technology; Derivatization; Detectors: Mass Spectrometry. **Extraction:** Solid-Phase Extraction. **Appendix: 2/Essential Guides to Method Development in Gas Chromatography. Essential Guides to Method Development in Solid-Phase Extraction.**

Further Reading

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